

D4  
55. (once amended) An isolated monomer of claim 52, wherein the monomer has a molecular weight of about 126 kDa, which is calculated from amino acid sequence of the monomer.

REMARKS

**I. Status of the Claims**

Claims 47, 49-52, and 54-56 are pending. The present amendment inserts in claims 50 and 55 the phrase "which is calculated from amino acid sequence of the monomer," referring to the method of determining molecular weight. This amendment is supported by the specification, e.g., on page 10 lines 23-25, where molecular weight is "predicted." A skilled artisan will understand that to "predict" molecular weight of a polypeptide one must perform a calculation based on amino acid sequence. In claims 47 and 52, the word "activity" is replaced by "amplitude." This amendment is supported by the specification, e.g., on page 14 lines 26-29. Furthermore, the word "approximately" is deleted from claims 47 and 52. The present amendment adds no new matter.

**II. Claim Rejections**

**A. 35 USC §112 Second Paragraph**

Claims 47, 49-52, and 54-56 were rejected under 35 USC §112 second paragraph for alleged indefiniteness. Applicants respectfully traverse the rejections.

***"Molecular weight"***

Claims 50 and 55 were rejected for the recitation of molecular weight of the pH sensitive potassium channel monomer of the present invention without indicating the method for determining molecular weight. As amended, the claims now recite that the molecular weight is calculated from amino acid sequence of the monomer. Applicants thus request the withdrawal of the rejections.

***"Activity"***

Claims 47 and 52 were rejected for the recitation of the term "activity." The Examiner alleged that it is unclear what activity is referred to and thus the metes and

bounds of the claims cannot be determined. The present amendment has replaced "activity" with "amplitude." The indefiniteness rejections are thus overcome.

*"Channel activity above approximately intracellular pH of 7.1"*

Claims 47 and 52 were rejected for the recitation of "channel activity above approximately intracellular pH of 7.1." The Examiner asserted that the word "approximately" makes unclear the metes and bounds of the claims. As amended, the word "approximately" has been deleted. Thus, the rejections should be properly withdrawn.

Taken together, the pending claims as amended have eliminated the basis of the indefiniteness rejections. Applicants submit that withdrawal of the rejections is proper.

**B. 35 USC §101 and 35 USC §112 First Paragraph**

Claims 47, 50-52, and 54-56 were rejected under 35 USC §101 for alleged lack of utility. The rejections have been maintained from a previous Office Action mailed October 23, 2001, despite the declaration by Dr. Timothy Jegla and Applicants' arguments. Specifically, the Examiner stated that the rejections cannot be withdrawn unless there is a showing that the potassium channel of the present invention is expressed in spermatocytes.

Applicants respectfully direct the Examiner's attention to page 58 lines 15-29. This section describes *in situ* hybridization experiments where labeled Slo3 probe was used to detect the expression of the potassium channel. In this experiment, testes from white mice more than 30 days old were dissected, frozen, and sectioned to make slides. A partial mSlo3 cDNA of about 1kb was used to make both sense and antisense RNA probes, which were labeled with <sup>32</sup>P and incubated with the slides. Labeling was observed in annular rings corresponding to the positions of spermatocytes in seminiferous tubules. The experiment results indicate Slo3 expression in spermatocytes. Moreover, the expression of Slo3 in mammalian spermatocytes is known to those skilled in the art as

it has been reported in peer-reviewed scientific publications, *see, e.g.*, page 1 in the "ABSTRACT" section and page 8 the "RESULTS" section of Schreiber et al., *J. Biol. Chem.* 273:3509-3516, 1998 (attached as Exhibit A). As such, Applicants respectfully request the withdrawal of the rejections under 35 USC §101.

The claims were also rejected under 35 USC §112 first paragraph. The Examiner alleged that one skilled in the art would not know how to use the invention when there is no established utility of the invention. In light of the foregoing discussion, the enablement rejections on the ground of lack of utility should be properly withdrawn.

**C. 35 USC §112 First Paragraph**

Claims 47, 49-52, and 54-56 were further rejected under 35 USC §112 first paragraph for alleged failure to meet the enablement requirement. Applicants respectfully traverse the rejections.

The Examiner first stated that the claimed invention is not properly enabled because no utility under 35 USC §101 has been established and one skilled in the art would not know how to use the invention. In light of the earlier discussion demonstrating a utility of the invention that is specific, substantial, and credible, Applicants submit that the enablement rejections based on lack of utility are overcome.

The Examiner also asserted that the specification does not provide the special technical features of the invention required for activity and that an artisan would not know how to use the invention in the case of variant potassium channels that are within the claimed genus but have different functions. Applicants cannot agree with the Examiner's assertion.

All pending claims recite a functional element: the claimed monomer forms a potassium channel having a unit conductance of 80-120 pS and having increased potassium channel current amplitude above intracellular pH of 7.1, when expressed in *Xenopus* oocyte. This functional element effectively excludes any and all polypeptides with sequence similarity to the claimed monomer yet without the specified functions (i.e.,

the inoperable variants) from the claim scope. The teaching of the present application will allow one with ordinary skill in the art to, according to the functional limitation recited by the pending claims, to screen for and eliminate inoperable embodiments using routine methodology.

According to MPEP §2164, the standard for adequate enablement is that the disclosure must allow one of ordinary skill in the art to make and use the invention without undue experimentation. The test for enablement is set forth in *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). The consideration of multiple factors is necessary: the breadth of the claims; the nature of the invention; the state of the prior art; the level of predictability in the art; the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In the present case, the claims are directed to pH sensitive potassium channel polypeptide monomers with a well-defined structure and readily testable functional feature. Working examples of mouse and human Slo3 are provided. The specification also contains ample directions to practice the invention, such as methods of cloning Slo3 nucleic acid sequences (see, e.g., page 29 line 26 to page 32 line 9; Examples I and V), expression of Slo3 nucleic acid sequences (see, e.g., page 32 line 12 to page 34 line 21; Example III), purifications of Slo3 polypeptides (see, e.g., page 34 line 24 to page 37 line 22), immunological detection of Slo3 (see, e.g., page 37 line 25 to page 44 line 9), and assays for Slo3 electrophysiological features (see, e.g., Example IV). The level of technical sophistication is high in the art, and the potassium channel variants can be readily tested according to the methods commonly used by those skilled in the art or the methods taught by the specification (such as nucleic acid hybridization and assays for electrophysiological characteristics) to eliminate inoperable embodiments. Such experimentation utilizes well-established techniques and is routinely conducted in the art. Thus, the experimentation does not constitute undue experimentation. MPEP §2164.01.

In summary, Applicants believe that the disclosure by the present application is sufficiently enabling for a person with ordinary skill in the art to practice the invention and that no undue experimentation is required. The rejections for inadequate enablement should thus be properly withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is urged. If the Examiner believes a telephone conference would aid in the prosecution of this case in any way, please call the undersigned at 415-576-0200.

Respectfully submitted,



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**APPENDIX A**

**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

47. (twice amended) An isolated polypeptide monomer of a pH sensitive potassium channel, the monomer:

- (i) forming a potassium channel having a unit conductance of 80-120 pS and having increased potassium channel current amplitude [activity] above [approximately] intracellular pH of 7.1, when the monomer is expressed in *Xenopus* oocyte; and
- (ii) encoded by a nucleic acid that specifically binds under stringent hybridization conditions to the complement of a nucleic acid encoding an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:16 or SEQ ID NO:18, wherein the hybridization reaction is incubated at 42°C in a buffer comprising 50% formamide, 5x SSC, and 1% SDS, and washed at 65°C in a buffer comprising 0.2x SSC and 0.1% SDS.

50. (once amended) An isolated monomer of claim 47, wherein the monomer has a [calculated] molecular weight of about 126 kDa, which is calculated from amino acid sequence of the monomer.

52. (twice amended) An isolated polypeptide monomer of a pH sensitive potassium channel, the monomer:

- (i) forming a potassium channel having a unit conductance of 80-120 pS and having increased potassium channel current amplitude [activity] above [approximately] intracellular pH of 7.1, when the monomer is expressed in *Xenopus* oocyte; and
- (ii) encoded by a nucleic acid that specifically binds under stringent hybridization conditions to the nucleic acid disclosed in SEQ ID NO:2, SEQ ID NO:4,

SEQ ID NO:17 or SEQ ID NO:19, wherein the hybridization reaction is incubated at 37°C in a buffer comprising 40% formamide, 1M NaCl, and 1% SDS, and washed at 45°C in a buffer comprising 1x SSC.

55. (once amended) An isolated monomer of claim 52, wherein the monomer has a [calculated] molecular weight of about 126 kDa, which is calculated from amino acid sequence of the monomer.



## APPENDIX B

### CLAIMS PENDING UPON ENTRY OF AMENDMENT

47. (twice amended) An isolated polypeptide monomer of a pH sensitive potassium channel, the monomer:

(i) forming a potassium channel having a unit conductance of 80-120 pS and having increased potassium channel current amplitude above intracellular pH of 7.1, when the monomer is expressed in *Xenopus* oocyte; and

(ii) encoded by a nucleic acid that specifically binds under stringent hybridization conditions to the complement of a nucleic acid encoding an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:16 or SEQ ID NO:18, wherein the hybridization reaction is incubated at 42°C in a buffer comprising 50% formamide, 5x SSC, and 1% SDS, and washed at 65°C in a buffer comprising 0.2x SSC and 0.1% SDS.

49. (as filed) An isolated monomer of claim 47, wherein the monomer has an amino acid sequence of SEQ ID NO:1, SEQ ID NO:16 or SEQ ID NO:18.

50. (once amended) An isolated monomer of claim 47, wherein the monomer has a molecular weight of about 126 kDa, which is calculated from amino acid sequence of the monomer.

51. (as filed) An isolated monomer of claim 47, wherein the monomer is a subunit of a homomeric potassium channel.

52. (twice amended) An isolated polypeptide monomer of a pH sensitive potassium channel, the monomer:

(i) forming a potassium channel having a unit conductance of 80-120 pS and having increased potassium channel current amplitude above intracellular pH of 7.1, when the monomer is expressed in *Xenopus* oocyte; and

(ii) encoded by a nucleic acid that specifically binds under stringent hybridization conditions to the nucleic acid disclosed in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:17 or SEQ ID NO:19, wherein the hybridization reaction is incubated at 37°C in a buffer comprising 40% formamide, 1M NaCl, and 1% SDS, and washed at 45°C in a buffer comprising 1x SSC.

54. (as filed) An isolated monomer of claim 52, wherein the monomer has an amino acid sequence of SEQ ID NO:1, SEQ ID NO:16 or SEQ ID NO:18.

55. (once amended) An isolated monomer of claim 52, wherein the monomer has a molecular weight of about 126 kDa, which is calculated from amino acid sequence of the monomer.

56. (as filed) An isolated monomer of claim 52, wherein the monomer is a subunit of a homomeric potassium channel.

J Biol Chem, Vol. 273, Issue 6, 3509-3516, February 6, 1998

## Slo3, a Novel pH-sensitive K<sup>+</sup> Channel from Mammalian Spermatocytes\*

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### ► ABSTRACT

Potassium channels have evolved to play specialized roles in both excitable and inexcitable tissues. Here we describe the cloning and expression of Slo3, a novel potassium channel abundantly expressed in mammalian spermatocytes. Slo3 represents a new and unique type of potassium channel regulated by both intracellular pH and membrane voltage. Reverse transcription-polymerase chain reaction, Northern analysis, and *in situ* hybridization show that Slo3 is primarily expressed in testis in both mouse and human. Because of its sensitivity to both pH and voltage, Slo3 could be involved in sperm capacitation and/or the acrosome reaction, essential steps in fertilization where changes in both intracellular pH and membrane potential are known to occur. The protein sequence of mSlo3 (the mouse Slo3 homologue) is similar to Slo1, the large conductance, calcium- and voltage-gated potassium channel. These results suggest that Slo channels comprise a multigene family, defined by a combination of sensitivity to voltage and a variety of intracellular factors. Northern analysis from human testis indicates that a Slo3 homologue is present in humans and conserved with regard to sequence, transcript size, and tissue distribution. Because of its high testis-specific expression, pharmacological agents that target human Slo3 channels may be useful in both the study of fertilization as well as in the control or enhancement of fertility.

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### ► INTRODUCTION

Many ion channels respond to membrane depolarization or intracellular ligands. In the Slo1 channel, originally cloned from *Drosophila*, these features are combined in a channel that opens in response to both depolarization and intracellular calcium increases (1, 2). Slo1 channels cloned from mouse and human show strong conservation of sequence and functional properties (3-7).

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One proposed role of the Slo1 channel is to provide negative feedback for the entry of calcium into cells via hyperpolarization-induced closure of voltage-dependent calcium channels. Perhaps because of the versatility of this mechanism, Slo1 channels are expressed in many tissues where voltage-gated calcium channels are present, including brain, skeletal and smooth muscle, auditory hair cells, pancreas, and adrenal gland (8-15). In contrast to other multigene voltage-gated K<sup>+</sup> channel families, Slo1 has remained the sole functionally characterized representative of its family (16). Here we describe mSlo3,<sup>1</sup> a pH- and voltage-dependent Slo family member prominently expressed in mouse spermatocytes. Despite similarity in sequence of mSlo3 to mSlo1, mSlo3 is insensitive to calcium over a wide concentration range. Thus, the unifying characteristics of the Slo gene family of channels may be a dual sensitivity to membrane voltage and a variety of intracellular factors, such as [H<sup>+</sup>] for mSlo3.

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The uniquely abundant expression of mSlo3 in developing spermatocytes presents further interesting questions. Spermatocytes require proteins tailored to fulfill roles unique to the process of germ cell development and fertilization. Cellular signaling in spermatocytes is tightly regulated to prevent inappropriate activation of the irreversible steps that prepare the sperm to fertilize the oocyte. Many of these steps are triggered and coordinated by changes in membrane potential and intracellular Ca<sup>2+</sup> concentration and pH. Because of the central importance of these events in development, many efforts have been made to identify the specific proteins, including ion channels, which regulate spermatocyte function. In particular, there have been reports of channels present in spermatocytes (19, 20), including voltage-dependent calcium channels (21-24). In addition, a cyclic nucleotide-gated channel has been directly cloned from testis (25). The mSlo3 channel exhibits a unique combination of voltage dependence and sensitivity to pH that may be important for spermatocyte function.

## ► MATERIALS AND METHODS

**Cloning**-- By tBlastn (NCBI), an EST (GenBank<sup>TM</sup> accession number [AA072586](#)) was identified by homology to the C-terminal "tail" of mSlo1 (17). The EST originated from a mouse promyelocytic WEHI-3 cell line cDNA library. A <sup>32</sup>P-labeled 1,254-bp PCR product generated from the EST pBluescript plasmid (Genome Systems) was employed to isolate cDNA clones from a WEHI-3 library (Stratagene) by hybridization. The oligonucleotides used to generate the probe were 5'-GTGGATGATACCGACATGCTGGAC-3' (sense) and 5'-GAGACCACCTCTCCCGTGTGCT-3' (antisense). mSlo3 expression in the WEHI-3 cell line is apparently anomalous; all isolated cDNAs were inappropriately spliced or truncated, and PCR analysis of the WEHI-3 cDNA bank using combinations of primers complementary to mSlo3 and vector sequence indicated that complete cDNAs were not represented. Subsequent screening of a mouse testis cDNA library (Dr. Graeme Mardon) yielded cDNAs that extended to the putative initiator methionine, as shown in Fig. 1. The reading frame was closed upstream from the initiator methionine. A full-length cDNA was constructed from two overlapping cDNAs. The presence of full-length transcripts in testis corresponding to this cDNA was verified by RT-PCR from total testis RNA. The entire cDNA was sequenced in both directions. For expression in *Xenopus* oocytes, a Kozak initiator sequence (26) was

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introduced by PCR, and the entire open reading frame was subcloned into the pOocyte-Xpress vector (17). cRNA was generated using the mMessage mMachine kit (Ambion); details of cRNA synthesis were as previously published (17).

**RT-PCR**-- For each tissue, Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) was used on 5.0  $\mu$ g of total RNA primed with 25  $\mu$ M random hexanucleotides (Boehringer Mannheim) and 200  $\mu$ M dNTPs at 42  $^{\circ}$ C for 1 h. 0.1% of each first strand synthesis was assayed by PCR using 1.0  $\mu$ M oligonucleotide primers, 200  $\mu$ M dNTPs, and 0.0075 units of KlenTaq, cycling 30 times. Reaction products were electrophoresed on 1.5 and 3.0% agarose gels using standard Tris-borate (TBE) buffer and visualized by staining with ethidium bromide. PCR primer pairs used were 1) mSlo3 (S4 to S5), 5'-CTCGAACTCCCTAAAATCTTACAGAT-3' (sense) and 5'-TTCCGTTGAGCCAGGGGTACCAAGAATT-3' (antisense) to generate a 156-bp product; 2) mSlo3 (S8 to S9), 5'-TCTGCTTGTAAAGCTAAATCT-3' (sense) and 5'-TTTCAAAGCCTCTTAGCGGTAA-3' (antisense) to generate a 690-bp product; 3) mSlo3 (S9 to S10), 5'-TTATGCCTGGATCTGCACTCTACATG-3' (sense) and 5'-ATAGTTCCGTCTACTACCGAAA-3' (antisense) to generate a 221-bp product; 4) human  $\beta$ -actin, 5'-GATGATATGCCGCGCTCGTCGAC-3' (sense) and 5'-TCGGTCCAGGTCTGCGTCCTACCGTAC-3' (antisense) to generate a 535-bp product.

**Northern Blot Analysis**-- Total RNA was isolated from freshly dissected mouse tissue using Trizol (Life Technologies, Inc.). 20  $\mu$ g of total RNA from each tissue was electrophoresed on a 1% agarose denaturing gel using MOPS-formaldehyde buffer then transferred to nitrocellulose. The human tissue blot was obtained commercially (CLONTECH). A PCR product generated using primers 5'-CGGAAACGTCATGTACAATCGAAATCCA-3' (sense) and 5'-TTCCGTTGAGCCAGGGGTACCAAGAATT-3' (antisense) was labeled using random hexanucleotides (Boehringer Mannheim). Both human and mouse blots were hybridized and washed under standard high stringency conditions and exposed to x-ray film for 4-16 h. After hybridization with mSlo3 probes, blots were rehybridized with a human  $\beta$ -actin probe to verify RNA loading.

**In Situ Hybridization**-- Testes from white mice >30 days old were dissected, frozen, sectioned immediately with a cryostat, collected on slides, and stored at -20  $^{\circ}$ C. A partial mSlo3 cDNA (approximately 1 kb, corresponding to coding sequence for residues 170-510) was subcloned into pBluescript II KS<sup>+</sup> (Stratagene). T3 and T7 RNA polymerase (Stratagene) were used to synthesize [<sup>33</sup>P] UTP-labeled antisense and sense probes, respectively, from linearized plasmid. Slides were hybridized overnight at 55  $^{\circ}$ C. After washing, slides were dipped in NTB-2 liquid emulsion (Eastman Kodak Co.), air-dried, and placed in light-protected boxes at 4  $^{\circ}$ C for 10 days.

**Electrophysiology**-- cRNA (40 nl at approximately 1  $\mu$ g/ $\mu$ l) was injected into mature *Xenopus* oocytes; recordings were made 1-8 days later. For whole cell recording, medium nd96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5) supplemented with 1 mM DIDS (to block endogenous chloride currents) was employed as a bath solution or modified as noted in the figure legends. Patches were perfused with either zero Ca<sup>2+</sup>-EGTA solutions (160 mM potassium gluconate, 34 mM KOH, 10 mM HEPES, and 10 mM EGTA) or Ca<sup>2+</sup>-containing (184 mM potassium gluconate,

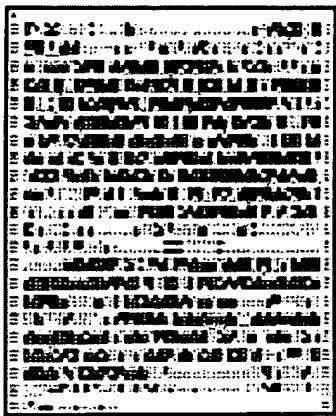
10 mM KOH, 10 mM HEPES, 200  $\mu$ M hemicalcium gluconate) solutions. HCl was used to adjust the pH. The pipette solution contained 0.5 mM potassium gluconate, 0.5 mM KCl, 1.1 mM KOH, 10 mM HEPES, 159 mM sodium gluconate, and 2 mM hemimagnesium gluconate, pH 7.1. Whole cell recordings were obtained using the two-electrode voltage clamp TEV-200 amplifier (Dagan). Patch currents were recorded on either an Axopatch 1B or 200A amplifier (Axon) and digitized at either 3.4, 5, or 10 kHz. Data acquisition and analysis programs were CCURRENT and CQUANT (Dr. Keith Baker) or pClamp6 (Axon). Single-channel conductance was determined from the slope of the unitary current amplitude *versus* voltage relation. The tail current amplitude *versus* voltage data (see Fig. 4) were fit with the sum of two independent Goldman-Hodgkin-Katz current equations. Each equation has the form  $I = PV_m F^2/RT \times ([S]_i - [S]_o \times \text{EXP}(-V_m F/RT))/1 - e^{-V_m F/RT}$ , where  $S$  is either  $K^+$  or  $Na^+$ .  $P$ , the permeability of  $Na^+$  or  $K^+$ , was allowed to vary freely.  $[S]_i$  and  $[S]_o$  represent ion concentrations inside and outside the oocyte, respectively;  $V_m$  is the membrane potential;  $F$ ,  $R$ , and  $T$  have their usual meanings. Reversal potential *versus* varying extracellular cation concentration (see Fig. 4) was fit with the Goldman-Hodgkin-Katz equation:  $E_{\text{reversal}} = RT/F \times \ln([K^+]_o + P \times [Na^+]_o)/([K^+]_i + P \times [Na^+]_i)$ , where  $P$  is the  $Na^+$  to  $K^+$  permeability ratio. Fits were performed using SigmaPlot (Jandel). Recordings were made at room temperature except tail currents, which were recorded at 11 °C using a Peltier device (Cambion).

## ► RESULTS

**Cloning and Primary Sequence of mSlo3--** We isolated the mSlo3 cDNA from a testis cDNA library based on its homology to the large conductance calcium-activated (BK) potassium channel, mSlo1 (see "Materials and Methods"). The probe was generated from an expressed sequence tag identified in the GenBank™ data base. The new channel was termed mSlo3 (the "m" prefix denoting mouse derivation, "h" referring to the human homologue, hSlo3). Fig. 1 illustrates that the 1,112 amino acid mSlo3 protein is similar along its entire length to the 1,169 amino acid mSlo1 protein (3) as well as *Drosophila* Slo1 (*dSlo1*) (1, 2). The hydrophilicity profiles of both sequences indicate 11 hydrophobic segments, S0 through S10 (Refs. 3 and 27 and Fig. 1B). As with mSlo1, these can be divided into core and tail domains. Similarity between mSlo3 and mSlo1 is greatest in the core domain. The mSlo3 core (S0 through S8: mSlo3 residues 35 through 641) shares 56 and 50% identity with mSlo1 and dSlo1 cores, respectively. (Interspecies homologues mSlo1 and dSlo1 share 62% identity.) The mSlo3 tail (S9 and S10: mSlo residues 686-1136) shares 39% identity with mSlo1 and dSlo1, respectively. (The interspecies homologues mSlo1 and dSlo1 share 68% identity in this region.) A linker region having no significant conservation is found between S8 and S9. More detailed comparison of mSlo3 and mSlo1 sequences implied two functional properties of mSlo3. 1) The absence of the "calcium bowl" (18) suggested that mSlo3 may be activated by factors other than  $Ca^{2+}$ . 2) In the  $K^+$ -selective pore, a GFG motif, rather than the typical GYG, suggested differences in ionic selectivity between the two channels (28, 29).

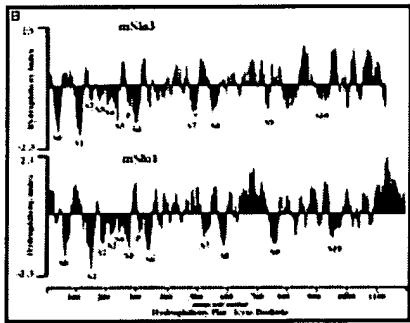
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**Fig. 1. Primary sequence of mSlo3.** *A*, alignment of the



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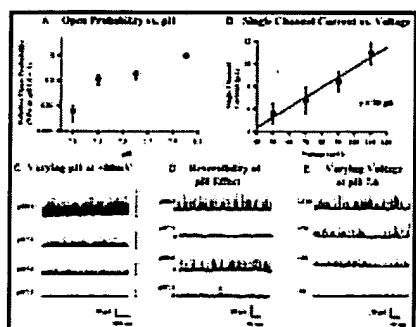
primary sequence of mSlo3 with BK  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels mSlo1 (mouse) and dSlo1 (*Drosophila*). Hydrophobic segments are designated S0 through S10 (3, 27). An *arrowhead* indicates a phenylalanine residue (F) in the pore region critical for ion selectivity (see text). The region designated "Calcium Bowl" has been implicated in the regulation of mSlo1 by calcium (18). The overall core and tail organization of Slo1 has been conserved in Slo3 (17). mSlo1 (mbr5) (3) and dSlo1 (splice variant A2C2E2G5I0) (1, 2) sequences are shown. Slo2, not shown, is a more distantly related sequence (16). *B*, Kyte-Doolittle (49) hydrophilicity plots of mSlo3 and mSlo1.



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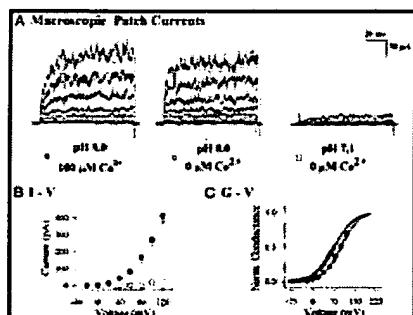
*Heterologous Expression of mSlo3 cRNA Produces Voltage- and pH-sensitive Currents--* When expressed in the *Xenopus* oocyte expression system, mSlo3 cRNA produced currents that were sensitive to both pH and voltage. This was demonstrated in observations of single channel behavior (Fig. 2), macroscopic currents in the patch configuration (Fig. 3), and whole cell oocyte currents recorded in the two-electrode voltage clamp mode (Fig. 4). In patches where unitary openings could be resolved, mSlo3 single-channel currents were observed to have very brief open times. Currents were sensitive to intracellular pH. Currents were small or absent at a  $\text{pH}_i$  of 7.1 or lower, whereas raising  $\text{pH}_i$  resulted in sharp increases in channel activity (Fig. 2). As shown in Fig. 2, the effect of changing pH was completely and repeatedly reversible. Macroscopic currents in the inside-out patch configuration behaved consistently with single channel behavior, despite channel rundown, which occurred over the course of minutes (Fig. 3). Patches exposed to pH 7.1 produced virtually no current. However, raising the pH to 8.0 resulted in macroscopic currents that responded to depolarization and exhibited little or no inactivation. In the whole cell configuration, mSlo3 currents resembled a voltage-dependent delayed rectifier and, consistent with macroscopic currents in the patch clamp configuration, showed little or no inactivation (Fig. 4). This behavior indicates that the internal pH of oocytes must be higher than pH 7.1, consistent with previous reports that reveal resting *Xenopus* oocyte pH near 7.5 (30, 31). Based on

this assumption, it was predicted that the manipulation of internal pH would either reduce the amplitude of observed whole cell currents (after acidification) or increase the amplitude of currents (after alkalization). Thus, acidification of the oocyte using bicarbonate-based bath solution (30) reduced the amplitude of currents (Fig. 4*A, middle*), whereas alkalization by ammonium chloride (31) increased the amplitude of mSlo3 currents even after attenuation by bicarbonate (Fig. 4*A, right*).



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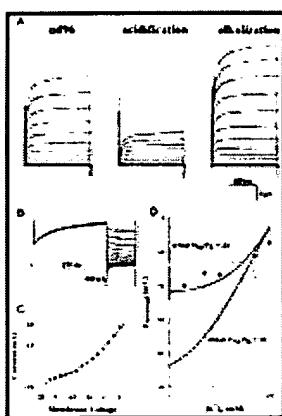
**Fig. 2. mSlo3 channel sensitivity to voltage and pH shown in inside-out patches.** *A*, open probability increases at higher pH at a constant voltage (+80 mV).  $NP_o$  was calculated assuming the presence of five channels in the patch. The data was normalized to the  $NP_o$  at pH 8. The cytoplasmic surface was exposed to recording solution at indicated pH ( $n = 3$ ). *B*, single-channel conductance calculated from the slope of single channel current amplitude plotted against membrane voltage in asymmetric  $K^+$  (cytoplasmic side: 194 mM  $K^+$ , 0 mM  $Na^+$ ; extracellular side: 2.1 mM  $K^+$ , 159 mM  $Na^+$ ;  $n = 4$ ). The conductance in symmetric 213 mM  $K^+$  was calculated to be 106 picosiemens (data not shown). *C*, currents from a single patch showing higher activity at higher pH; the open times of single channel events are brief. *D*, reversibility of pH effect. A single patch is shown alternately exposed to pH 8.0 and pH 7.1. The sequence is from top to bottom. *E*, channel activity increases at positive potentials at a constant pH (pH 7.6).



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**Fig. 3. Macroscopic current traces showing sensitivity to pH but insensitivity to calcium ion.** *A*, current traces from the same patch exposed to pH 8 with  $Ca^{2+}$  (filled circles), pH 8 without  $Ca^{2+}$  (triangles), or pH 7.1 without  $Ca^{2+}$  (squares). *B*, corresponding current-voltage ( $I$ - $V$ ) relations for the traces in *A*. *C*, calculated G-V curves for the patch in *A* exposed to 100  $\mu M$   $Ca^{2+}$  (filled circles) or 0  $Ca^{2+}$  (open triangles), both at pH 8.0. For comparison, the averaged whole-cell conductance-voltage relation was plotted (open diamonds;  $n = 12$ ). This relation falls approximately 25 mV to the left of the macroscopic patch curve. Each set of points was fitted with a single Boltzmann equation. Because none of the data points approach roll-over (saturation of the conductance), the Boltzmann fits must be regarded as an estimate only. The current shown in 0  $\mu M$   $Ca^{2+}$  at pH 7.1 (*A*) was restored in amplitude when the patch was again exposed to pH 8. The slight decrease in current amplitude between the two sets of

traces at pH 8 was most likely due to current rundown. Holding potential was  $-40$ ; on-line leak subtraction was employed.



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**Fig. 4. mSlo3 whole cell currents from *Xenopus* oocytes.** *A*, manipulation of intracellular pH alters current amplitude. *Left*, control currents at start of experiment. *Middle*, diminished current amplitude after intracellular acidification (12.5-min perfusion with  $\text{NaHCO}_3$  replacing  $\text{NaCl}$  in nd96). *Right*, recovery during alkalinization (10-min perfusion with nd96 supplemented with 30 mM  $\text{NH}_4\text{Cl}$ ). Voltage families are from  $-80$  to  $+60$  mV in 10-mV increments. *B*, representative tail currents used to determine channel selectivity. *C*, tail current amplitude *versus* voltage plotted for the currents shown in *B*. Instantaneous currents at the time of the voltage jump were calculated from exponential fits of tail currents extrapolated back to time zero. *Filled squares* indicate currents plotted *versus* test potential. A Goldman-Hodgkin-Katz current equation was fitted (*solid line*) to the tail current-voltage relation. The fit showed a  $\text{Na}^+/\text{K}^+$  permeability ratio of 0.15. The calculated underlying  $\text{K}^+$  (*triangles*) and  $\text{Na}^+$  (*open squares*) currents are also shown. (For the example shown in *B* and *C*, bath ion concentrations were 10 mM  $[\text{K}^+]$ , 88 mM  $[\text{Na}^+]$ , 11 °C.) *D*, reversal potentials plotted at different potassium concentrations to illustrate the relatively low selectivity of mSlo3 for  $\text{K}^+$  over  $\text{Na}^+$ . mSlo3 data is shown compared with the more highly selective dShab channel (50). Reversal potentials were determined by measuring tail currents in varying external  $[\text{K}^+]$ ;  $[\text{Na}^+]$  was also varied, so that the total monovalent concentration ( $[\text{K}^+] + [\text{Na}^+]$ ) was 98 mM. Points were fitted with a Goldman-Hodgkin-Katz equation where the  $\text{Na}^+/\text{K}^+$  permeability ratio,  $P$ , was allowed to vary freely. For mSlo3 reversal potentials at 2, 5, 10, 50, and 98  $[\text{K}^+]$ ,  $n = 6, 3, 5, 6, 7$ , respectively; for dShab at 2, 10, 50, and 98,  $n = 3, 1, 3, 3$ .

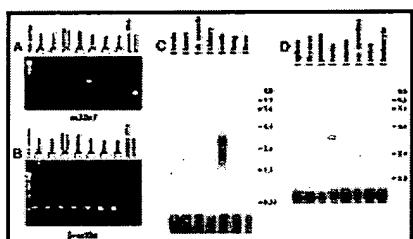
The effect of calcium ion on the gating of mSlo3 currents was investigated by observing macroscopic currents in the inside-out patch configuration and exposing the cytoplasmic surface to various concentrations of  $\text{Ca}^{2+}$ . It was found that macroscopic patch currents were unaffected by changing  $[\text{Ca}^{2+}]$  from nominally zero (10 mM EGTA solution with no added calcium) to 100  $\mu\text{M}$   $\text{Ca}^{2+}$  perfused on the intracellular face (Fig. 3). As a control, the same solutions were used on inside-out patches containing mSlo1 channels instead of mSlo3. Using an identical voltage protocol, large macroscopic currents were evoked at 100  $\mu\text{M}$ , but currents were absent in zero calcium conditions (data not shown; see also Ref. 18).

mSlo3 single-channel conductance was observed to be 90 picosiemens as measured in asymmetric

potassium concentrations (Fig. 2) and 106 in symmetric high  $K^+$  (data not shown). This is considerably lower than that seen for mSlo1 (270 picosiemens) measured in symmetric high  $K^+$  (3). Unlike mSlo1, mSlo3 is relatively insensitive to tetraethylammonium. mSlo3 channels were tested for external TEA sensitivity in the whole cell, two-electrode recording mode. Oocytes expressing mSlo3 cRNA were stepped from  $-40$  to  $+80$  mV while several concentrations of TEA were added sequentially to the bath. A plot of current *versus* TEA concentration at  $+60$  mV revealed an  $EC_{50}$  of 49 mM (data not shown). In contrast, mSlo1 is approximately 350-fold more sensitive (having an  $EC_{50}$  of 0.14 mM) (3).

*Slo3 Currents Show Relaxed  $K^+$  Selectivity*-- One conspicuous difference in sequence between mSlo3 and mSlo1 occurs in a region implicated in ion selectivity. All channels with high selectivity for potassium over sodium have a GYG sequence motif in the "P" region (28, 29). In contrast, mSlo3 has a GFG at this location (Fig. 1, residue 279). It was previously shown in a Shaker  $K^+$  channel that substitution of F (Phe) for Y (Tyr) in the GYG sequence decreased selectivity for  $K^+$  over  $Na^+$  (32). This suggested that mSlo3 could have less selectivity for  $K^+$  over  $Na^+$  than most potassium channels. To test this possibility, we analyzed the reversal potential for mSlo3 current tails at various concentrations of external potassium and sodium ion (Fig. 4). Our results showed that mSlo3 is less selective for  $K^+$  over  $Na^+$  than Slo1, having a  $P_K/P_{Na}$  of approximately 5 *versus*  $>50$  for Slo1 (3, 5).

*Slo3 Expression Is Prominent Only in Spermatocytes*-- Figs. 5, A and B, show results from RT-PCR performed on RNA from brain, skeletal muscle, lung, liver, kidney, and heart; only testis produced a positive signal. Northern blots using total RNA from the same tissues gave a robust signal after only 4 h of exposure. A transcript of approximately 4 kb was seen only in testis (Fig. 5). A longer exposure (18 h) failed to reveal bands from any additional tissues (data not shown).



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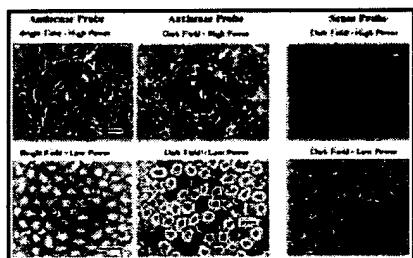
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**Fig. 5. Expression of mSlo3 transcripts is largely restricted to the testis.** A, RT-PCR of mouse brain, heart, skeletal muscle, kidney, testis, lung, and liver with mSlo3-specific primers, with (+) and without (−) the addition of reverse transcriptase. An expected 156-bp product is detected only in testis RNA. Similar results were obtained with two additional mSlo3 primer pairs specific to S8-S9 and S9-S10 regions (data not shown). B, control RT-PCR assays with  $\beta$ -actin-specific primers produce an expected 537-bp product from all tissues. Additional negative controls with genomic DNA (10 ng) or primers alone are also shown. C, Northern blot analysis of total RNA (20  $\mu$ g) from mouse brain, heart, skeletal (sk) muscle, kidney, testis, lung, and liver reveals an abundant mSlo3 transcript only in testis, with an approximate size of 4 kb. D, Northern blot analysis of polyadenylated RNA (2  $\mu$ g) from human tissues (spleen, thymus, prostate, testis, uterus, small (sm) intestine, colon, and leukocytes) with mSlo3 reveals a hybridizing mRNA species only in testis,

with an approximate size of 4 kb. Controls are shown below; the same blots were hybridized with a human  $\beta$ -actin probe.

**A Human Slo3 Homologue Is Present in Testis**-- Northern analysis of a human tissue blot also revealed a band at 4 kb, indicating the presence of a human Slo3 homologue of a conserved transcript size. The human Northern blot similarly indicated a restricted tissue distribution (Fig. 5). The small message size is unusual for a gene encoding such a large protein. Apparently, there may be as little as 700 bp of 5'- and 3'-untranslated regions. This small message size contrasts with the larger message size of mSlo1, which is more than twice as large in brain (3). Preliminary sequence data indicates hSlo3 is highly conserved at the molecular level.

**In Situ Hybridization**-- In testis, *in situ* hybridization revealed that mSlo3 message is expressed in the seminiferous tubules with the signal directly over developing spermatocytes (Fig. 6). The message is most abundant in the inner segments of these rings, corresponding to positions over maturing spermatocytes and possibly to early spermatids. The outermost regions of these rings contain spermatogonia, the stem cell from which spermatocytes are derived. The absence of hybridization (arrow in Fig. 6) in the peripheral areas suggests that expression is restricted to later stages of spermatogenesis. The absence of message in the interstices between rings also suggests that the nerves, blood vessels, and connective tissue in these regions of the testis probably lack expression of mSlo3.



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**Fig. 6. *In situ* hybridization localizes expression of mSlo3 mRNA in seminiferous tubules.** *Upper row*, high magnification light micrographs of adult mouse testis. *Left*, annular cross-section of a seminiferous tubule (arrow), containing mostly developing spermatocytes (S). Spermatogerm cells and primary spermatocytes are at the outer edges, whereas more mature spermatocytes are found near the lumen (L). Less abundant Sertoli and Leydig cells are difficult to distinguish. *Middle*, the same field shows intense hybridization signal (white puncta) in more mature spermatocytes. The interstices between tubules are unlabeled. *Right*, control section hybridized with an mSlo3 sense probe. *Lower row*, low magnification micrographs. *Left*, many seminiferous tubule cross-sections are visible. *Middle*, dark field of a similar section shows labeling of annular masses of developing spermatocytes. Scattered tubules with low hybridization signal may reflect segments at earlier stages of spermatogenesis. *Right*, a similar control section hybridized with sense probe. *Scale bar: top, 100  $\mu$ m; bottom, 500  $\mu$ m.*

## ► DISCUSSION

Sensitivity to both intracellular alkalinization and membrane depolarization

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distinguishes mSlo3 from other cloned channels. Previous reports demonstrate that other channels from various tissues are sensitive to pH, but their functional properties as well as their tissue distributions differ from mSlo3. One example is the acid-sensing channel (ASIC), which opens upon intracellular acidification (33). These channels are present in dorsal root ganglia and are involved in pain sensation. The ASIC channel is in the amiloride-sensitive  $\text{Na}^+$  channel degenerin family and is not similar to  $\text{K}^+$  channels. In contrast, TASK and RACTK1 are in the  $\text{K}^+$  channel superfamily but lack the S4 voltage sensor and do not respond to membrane potential changes. TASK is a recently cloned, four-transmembrane domain channel responsive to extracellular proton concentrations in the physiological range; it is presumed that the TASK channel contributes to the resting  $\text{K}^+$  conductance (34). The RACTK1 kidney  $\text{K}^+$  channel, a two transmembrane domain channel, is activated by intracellular alkalization but is voltage-insensitive (35). In contrast, mSlo3 as well as other Slo family channels may have evolved to respond to both voltage as well as other intracellular factors; calcium in the case of mSlo1 and pH in the case of mSlo3.

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The properties of mSlo3 make it difficult to work with. In addition to its very short mean open time and low open probability ( $P_o$ ) at physiological voltages, there is also a nagging rundown problem to contend with. It is especially difficult to get patches with sufficient channels for macroscopic current analysis. Because very large depolarizations are required to elicit activity of the channel,  $g/g_{\max}$  plots do not approach saturation and the Boltzmann fitted to these plots can only serve as an estimate of channel behavior. However, from these approximate Boltzmanns, the whole-cell current  $V_{50}$  appears to be  $+72 \pm 4.9$  mV (mean  $\pm$  S.E.;  $n = 12$ ; Fig. 3C). The slope of these  $V$  versus  $g/g_{\max}$  relations is extremely shallow (approximately 16 mV/e-fold). Thus, the base of the curve is in a physiological range even though  $g_{\max}$  is not attained until +200 mV. Because of this shallow slope, it appears that at least some channels must be open in the physiologically relevant voltage range. Perhaps the mSlo3 channel is designed to provide small increments of current. Alternatively, our heterologous expression system might be missing something that affects gating properties, perhaps a cytoplasmic factor or auxiliary subunit native to sperm that contributes to the gating of this channel. One hint that cytoplasmic factors may be important is the fact that the estimates of  $V_{50}$  from whole cell voltage clamp recordings are shifted approximately -25 mV relative to the macroscopic patch current (Fig. 3C). This may suggest that another factor does indeed influence channel gating. A missing factor could permit the mSlo3 channel to function more prominently within the pH and voltage range present in spermatocytes or mature sperm.

The mechanism of proton sensitivity in mSlo3 is unknown. In ROMK inward rectifier channels, a titratable lysine residue has been shown to confer reversible proton block when pH is reduced below 7.5, both in whole cell recordings from oocytes exposed to bicarbonate-containing media or in perfused detached patches (30, 36). It is possible that mSlo3 is inhibited by protons by a similar mechanism. In Slo1 channels, raising  $[\text{Ca}^{2+}]_i$  increases channel activity by enabling the channel to activate at more hyperpolarized voltages (8, 9). Preliminary data from mSlo3 suggests that changing the proton

concentration may not alter the voltage range of activation of the channels. Although Slo1  $\text{Ca}^{2+}$  sensitivity is altered at different  $\text{pH}_i$ , this effect may depend on the alteration of calcium affinity by protonating residues involved in calcium binding (37). It is not clear if or how this may relate to mSlo3 gating.

mSlo3 represents a new member of the Slo family of channels. Surprisingly, although these channels share a great deal of sequence identity, the functional features of these channels, exceptionally large conductance, calcium sensitivity, and high selectivity for  $\text{K}^+$  over  $\text{Na}^+$ , do not appear to be highly conserved Slo family characteristics. The structure of the Slo1 channel resembles a voltage-dependent channel core with an appended tail region that plays a modulatory role in gating by sensing  $\text{Ca}^{2+}$  (17, 18). We previously demonstrated the existence of two calcium sensing sites in the mSlo1 channel, the calcium bowl, a high affinity site in the tail region, and a lower affinity site at a location that has not yet been determined (18). The lack of  $\text{Ca}^{2+}$  sensitivity in mSlo3 implies that neither of these two sites are present. Properties associated with the core domain also differ between Slo3 and Slo1. Slo3 is approximately 350-fold less sensitive to external TEA. Underlying this difference may be the absence of a tyrosine residue at a site reported to be critical to external TEA sensitivity (38). Rather than a tyrosine residue as in TEA-sensitive channels, the mSlo3 sequence has a valine at the corresponding position (valine at position 283). The highly selective toxins that block BK channels, charybdotoxin (39) and iberiotoxin (40), did not affect mSlo3 currents at 50 and 20 nM, respectively (data not shown). In contrast, an S4 voltage-sensing domain is present in both Slo1 and Slo3, corresponding to voltage sensitivity, which the channels share (41). The pairing of voltage sensitivity with sensitivity to a variety of intracellular factors may be the unifying feature of the Slo family.

Several observations indicate uniquely abundant expression in testis: a high density of labeling in *in situ* experiments, a strong signal in Northern analysis using total RNA, and high representation in a testis cDNA library ("Materials and Methods"). These data cannot exclude very low levels of expression or spatially restricted patterns of expression within the other tissues examined. However, if mSlo3 expression is indeed largely restricted to spermatocytes, it may be that the pairing of sensitivity to pH and voltage is designed to fulfill a unique role in spermatocytes. All proteins utilized by the mature sperm are synthesized during spermatogenesis, as mature sperm lack translational activity. Thus, although the mSlo3 protein has not yet been identified in mature sperm, robust transcription in developing spermatocytes makes it likely that the channel is present at these later stages. The unlikely alternative is that mSlo3 is utilized only during a narrow window of time in sperm development. Assuming its presence in mature sperm, the unusually high permeability ratio of sodium to potassium could allow multiple roles for mSlo3 channels that depend on the extracellular environment that sperm encounter. In high external  $\text{Na}^+$  or  $\text{K}^+$ , Slo3 channels could have a depolarizing influence; conversely, where these ions were low in the external environment, the open channel would be hyperpolarizing. It is intriguing to speculate on the role of mSlo3, since both alkalization and depolarization are components of the signaling pathway during both sperm capacitation and the acrosome reaction, two essential steps preceding sperm fertilization of the oocyte (21, 42-45). Between mating and fertilization, sperm undergo capacitation, a process that later enables them to fertilize the oocyte. Capacitation involves an increase in cytosolic pH ( $\text{pH}_i$ ), which promotes metabolic and swimming activity (42, 46-47). An increase in  $\text{pH}_i$ ,

changes in membrane potential, and a rise in cytoplasmic  $[Ca^{2+}]$  trigger the acrosome reaction upon contact with the oocyte (21, 43, 48). As the sperm membrane depolarizes, voltage-gated calcium channels open, permitting the entry of calcium and thereby triggering the release of the acrosomal granule (23). Although the role of Slo3 in these processes remains speculative at this time, it is plausible that this channel plays a role in coordinating these events by directly linking cellular pH and membrane voltage.

Future investigation may focus on substances known to affect sperm function, which may use this channel as a target. Finally, agents that block or open this channel may be useful in the study or control of fertility.

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## ► FOOTNOTES

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank<sup>TM</sup>/EMBL Data Bank with accession number(s) AF039213.

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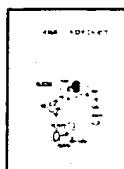
<sup>1</sup> The abbreviations used are: mSlo3, mouse Slo3; RT-PCR, reverse transcription-polymerase chain reaction; MOPS, 4-morpholino propanesulfonic acid; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; TEA, tetraethylammonium; bp, base pairs(s); kb, kilobase(s).

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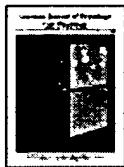
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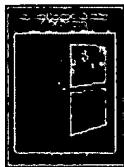
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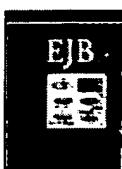
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